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# Specific integrin $\alpha$ and $\beta$ chain phosphorylations regulate LFA-1 activation through affinity-dependent and -independent mechanisms

Susanna C. Fagerholm, Tiina J. Hilden, Susanna M. Nurmi, and Carl G. Gahmberg

Division of Biochemistry, Faculty of Biosciences, University of Helsinki, FIN-00014 Helsinki, Finland

Integrins are adhesion receptors that are crucial to the functions of multicellular organisms. Integrin-mediated adhesion is a complex process that involves both affinity regulation and cytoskeletal coupling, but the molecular mechanisms behind this process have remained incompletely understood. In this study, we report that the phosphorylation of each cytoplasmic domain of the leukocyte function-associated antigen-1 integrin mediates different modes of integrin activation.  $\alpha$  Chain phosphorylation on Ser1140 is needed for conformational changes in the

integrin after chemokine- or integrin ligand-induced activation or after activation induced by active Rap1 (Rap1V12). In contrast, the  $\beta$  chain Thr758 phosphorylation mediates selective binding to 14-3-3 proteins in response to inside-out activation through the T cell receptor, resulting in cytoskeletal rearrangements. Thus, site-specific phosphorylation of the integrin cytoplasmic domains is important for the dynamic regulation of these complex receptors in cells.

## Introduction

The heterodimeric cell surface receptors called integrins are exceptional in that they can function as bidirectional signaling devices, regulating cell adhesion and migration after so-called inside-out signaling, and they can also signal into the cell to regulate growth, differentiation, and apoptosis after ligand binding (Giancotti and Ruoslahti, 1999; Hynes, 2002). The relatively small intracellular domains of integrins are involved in regulating signaling functions. Recently, separation of integrin cytoplasmic domains has been postulated as a mechanism of regulating integrin bidirectional signaling (Vinogradova et al., 2002; Kim et al., 2003; Tadokoro et al., 2003). Proximal events in the regulation of integrin activation and outside-in signaling presumably involve the binding of cytoplasmic molecules to the intracellular tails (Calderwood, 2004).

Dynamic adhesion is especially important in the immune system, where cells need to attach and detach continuously. The leukocyte function-associated antigen-1 (LFA-1) integrin ( $\alpha$ L $\beta$ 2 or CD11a/CD18) is expressed exclusively in leukocytes and is of fundamental importance to the function of the

immune system (Springer, 1990; Gahmberg, 1997). LFA-1 mediates cell adhesion under various conditions, e.g., during immunological synapse formation between the T cell and the antigen-presenting cell and during leukocyte emigration from the bloodstream into tissues. Whereas T cell receptor (TCR)-mediated adhesion is slow and sustained, chemokine-induced adhesion is fast and rapidly reversible. Both affinity-dependent and -independent mechanisms have been postulated as being important in the regulation of integrin activation (van Kooyk and Figdor, 2000; Carman and Springer, 2003; Calderwood, 2004). These mechanisms are not mutually exclusive, and different modes of integrin activation may involve different mechanisms working alone or together. For example, TCR-induced activation of LFA-1 has not been shown to involve affinity regulation (conformational changes) in the integrin, but instead has been closely correlated with the spreading phenotype of T cells and actin cytoskeleton rearrangements (Stewart et al., 1996, 1998). In contrast, chemokines mediate rapid conformational changes in LFA-1, as measured by activation epitope expression with mAbs and the measurement of soluble ligand binding to the integrin (Weber et al., 1999; Constantin et al., 2000). Chemokine-induced adhesion also involves the clustering of integrins (Constantin et al., 2000). Ligands can also induce conformational changes and clustering of integrins (Cabanas and Hogg, 1993; Li et al., 1995; Kotovuori et al., 1999; Kim et al., 2004).

S.C. Fagerholm and T.J. Hilden contributed equally to this paper.

Correspondence to Carl G. Gahmberg: carl.gahmberg@helsinki.fi

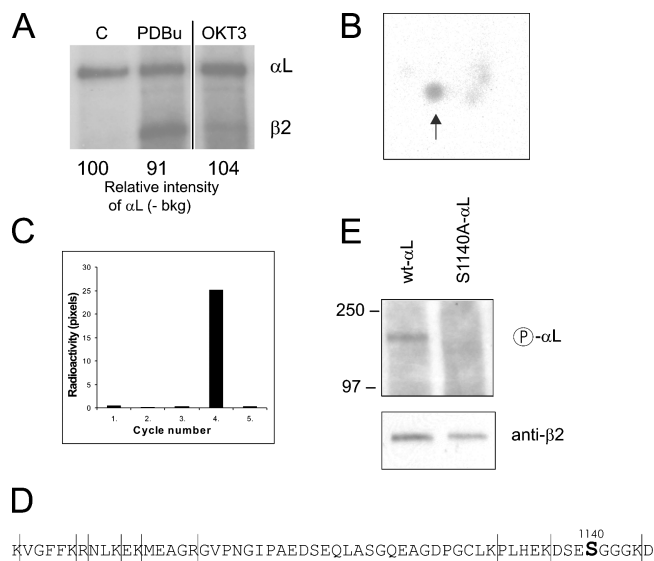
Abbreviations used in this paper: ICAM, intercellular adhesion molecule; J, Jurkat; LFA-1, leukocyte function-associated antigen-1; sICAM, soluble ICAM; TCR, T cell receptor; wt, wild-type.

Phosphorylation is a common mechanism for the regulation of surface receptor function and has also been reported in integrins, but its role in integrin regulation has remained only partially understood (Fagerholm et al., 2004). LFA-1 is phosphorylated on both the  $\alpha$  and  $\beta$  chains, with the  $\alpha$  chain being constitutively phosphorylated, whereas  $\beta$  chain phosphorylation becomes detectable after inside-out stimulation of the integrin (Hara and Fu, 1986; Chatila et al., 1989; Valmu and Gahmberg, 1995). The  $\alpha$  chain phosphorylation sites have not been mapped, and their functions are completely unknown. In contrast, the  $\beta$  chain phosphorylation sites are known (Hibbs et al., 1991; Fagerholm et al., 2002b; Hilden et al., 2003). The main phosphorylation site after phorbol ester stimulation of cells is Ser756, but this site is not involved in regulating adhesion (Hibbs et al., 1991). The threonine triplet (Thr758–760) in the  $\beta$ 2 chain is important for adhesion, interactions with the actin cytoskeleton, and modulation of cell spreading (Hibbs et al., 1991; Peter and O'Toole, 1995). Interestingly, threonine phosphorylation of the  $\beta$  chain has been reported (Valmu and Gahmberg, 1995) and threonine-phosphorylated integrins distribute preferentially to the actin cytoskeleton in cells (Valmu et al., 1999a). Additionally, it has been shown that 14-3-3 proteins from cell lysates interact with a Thr758-phosphorylated  $\beta$ 2 integrin peptide in vitro (Fagerholm et al., 2002b), but whether the interaction occurs in vivo or plays a role in adhesion has not been discovered. In this study, we investigated the role of both  $\alpha$  and  $\beta$  chain phosphorylations in the regulation of LFA-1-mediated adhesion.

## Results

### $\alpha$ L is phosphorylated on Ser1140 in T cells

To establish the role of LFA-1 phosphorylation in integrin regulation, we first mapped the phosphorylation sites in  $\alpha$ L. Endogenous  $\alpha$ L is phosphorylated in both resting  $^{32}$ P-labeled T cells and in cells activated by phorbol ester stimulation or through ligation of the TCR with the antibody OKT3 (Fig. 1 A). The labeled protein band was subjected to trypsin cleavage and phosphopeptide mapping, and only one major spot (radioactive peptide) was detected (Fig. 1 B). The maps from resting cells and from phorbol ester-activated cells were identical (unpublished data), thus no additional sites were phosphorylated after cell activation. Identification of the serine phosphorylation site was made using manual Edman degradation. The radioactive phosphorylated amino acid was detected as the fourth residue in the tryptic  $\alpha$ L peptide (Fig. 1 C). This result indicates that Ser1140 in the  $\alpha$ L cytoplasmic domain is the target of phosphorylation (Fig. 1 D). To confirm this, we substituted Ser1140 with Ala. COS-1 cells were transiently transfected with wild-type (wt)  $\alpha$ L and S1140A- $\alpha$ L, together with wt  $\beta$ 2, and cells were labeled with  $^{32}$ P. The LFA-1 protein was immunoprecipitated from cell lysates and subjected to SDS-PAGE analysis and autoradiography. No radioactive labeling was detected in S1140A- $\alpha$ L transfectants (Fig. 1 E). These data demonstrate that Ser1140 is the principal  $\alpha$ L phosphorylation site.

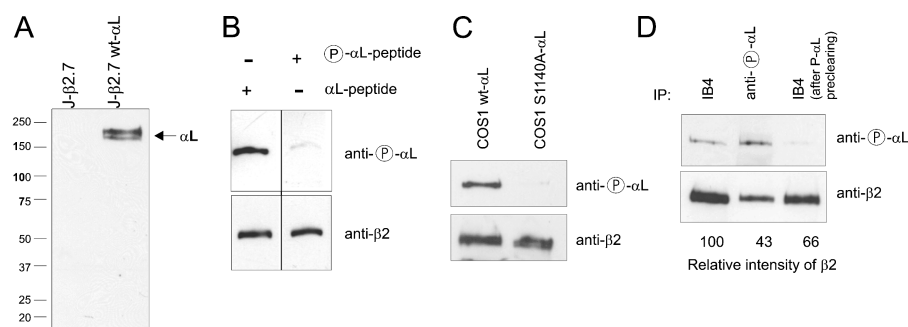


**Figure 1. Ser1140 is the phosphorylation site in the  $\alpha$ L chain of LFA-1.** (A) The integrin  $\beta$  chain is phosphorylated only after cell stimulation, whereas the  $\alpha$  chain is constitutively phosphorylated. Endogenous integrins were immunoprecipitated from control cells (C) or okadaic acid-pretreated (1.5  $\mu$ M), PDBu-activated (200 nM; 30 min), or OKT3-activated (10  $\mu$ g/ml; 60 min)  $^{32}$ P-labeled human T cells and analyzed by SDS-PAGE and autoradiography. The radiolabeled  $\alpha$ L bands were also quantified. (B) Phosphopeptide map of  $^{32}$ P-labeled  $\alpha$ L digested with trypsin from resting T cells. (C) The main spot in B (arrow) was subjected to Edman degradation. The radioactive phosphorylated amino acid was detected as the fourth residue in the tryptic  $\alpha$ L peptide. (D) Sequence of the  $\alpha$ L cytoplasmic domains with the Ser1140 phosphorylation site shown in bold. The trypsin cleavage sites in  $\alpha$ L are indicated with vertical lines. (E) COS-1 cells were transfected with wt  $\alpha$ L or S1140A- $\alpha$ L, together with wt  $\beta$ 2, and  $^{32}$ P-labeled LFA-1 was immunoprecipitated and analyzed by SDS-PAGE and autoradiography. No radioactive band was detected in S1140A- $\alpha$ L transfectants.  $\beta$ 2 integrins were detected from the same filter by immunoblotting to demonstrate equal loading.

### Ser1140 is phosphorylated at a high stoichiometry in resting T cells

To estimate the stoichiometry of  $\alpha$ L phosphorylation in leukocytes, we generated a phosphospecific antibody capable of recognizing  $\alpha$ L only when it has been phosphorylated on Ser1140. The antibody was  $\alpha$ L specific because it failed to react with lysates of Jurkat (J)- $\beta$ 2.7 cells, which are an  $\alpha$ L-deficient Jurkat cell line, and reacted with the  $\alpha$ L integrin in  $\alpha$ L-transfected J- $\beta$ 2.7 cells (Fig. 2 A). This antibody was also tested for recognition of immunoprecipitated LFA-1 from T cells in the presence of blocking  $\alpha$ L unphosphopeptide or phosphopeptide (Fig. 2 B). This phosphospecific antibody recognized  $\alpha$ L in the presence of competing unphosphorylated peptide (to block any interactions mediated by antibodies that recognize nonphosphorylated forms of the integrins), but in the presence of phosphopeptide no recognition occurred. Specificity was also confirmed by Western blotting of immunoprecipitated  $\alpha$ L from cells expressing wt  $\alpha$ L and from cells where Ser1140 had been mutated to nonphosphorylatable Ala (Fig. 2 C). The antibody reacted with wt  $\alpha$ L, but not with S1140A-mutated  $\alpha$ L. Thus, the experiments demonstrated that the antibody is sequence- and phosphospecific.

We used this phosphospecific  $\alpha$ L antibody to assess the stoichiometry of  $\alpha$ L phosphorylation. The total amount of



**Figure 2. Characterization of the phospho-specific  $\alpha$ L-Ser1140 antibody and assessment of the stoichiometry of  $\alpha$ L phosphorylation.** (A) A Ser1140 phosphospecific antibody was made, as described in Materials and methods, and used in a Western blot of cell extracts prepared from J- $\beta$ 2.7 cells and wt  $\alpha$ L-transfected J- $\beta$ 2.7 cells. (B) The antibody was tested for recognition of immunoprecipitated LFA-1 from T cells in the presence of 25  $\mu$ g/ml of blocking unphosphopeptide or 25  $\mu$ g/ml of phosphopeptide. An immunoprecipitation was made with the IB4 antibody, which recognizes only heterodimeric forms of the  $\beta$ 2 integrin.

The blots were stripped and reprobed with anti- $\beta$ 2 integrin antibodies (R2E7B) to demonstrate equal loading. (C) wt  $\alpha$ L and S1140A- $\alpha$ L integrins were immunoprecipitated from transiently transfected COS-1 cells, and phosphorylated  $\alpha$ L was detected by Western blotting. (D) The stoichiometry of the  $\alpha$ L phosphorylation was estimated using the phosphospecific  $\alpha$ L-Ser1140 antibody. The T cell lysates were immunoprecipitated with either the phosphospecific  $\alpha$ L antibody or IB4. The remaining  $\alpha$ L from phosphospecific  $\alpha$ L immunoprecipitation was immunoprecipitated by IB4. Immunoprecipitates were resolved by SDS-PAGE and phosphorylated  $\alpha$ L was detected.  $\beta$ 2 detection was used to demonstrate the total amount of integrin in samples. In the Western blot, the phosphospecific antibody was used in the presence of unphosphorylated peptide.

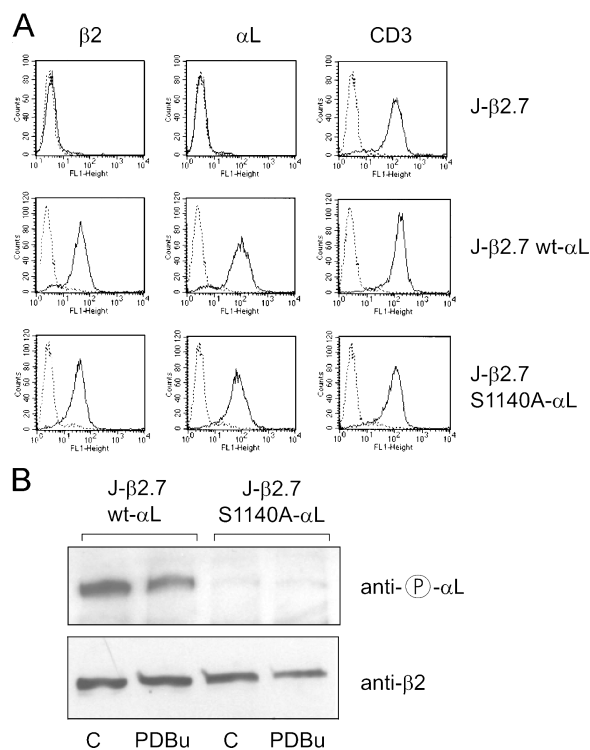
heterodimeric LFA-1 (100%) in T cell lysates was determined by immunoprecipitation with IB4, an antibody that recognizes heterodimeric LFA-1 rather than the monomeric  $\beta$ 2 (Fig. 2 D; Wright et al., 1983). The phosphospecific antibody immunoprecipitated  $\sim$ 40% of the total heterodimeric  $\alpha$ L from resting T cells (Fig. 2 D). The remaining  $\alpha$ L from this lysate was immunoprecipitated with IB4, but no  $\alpha$ L phosphorylation was seen in this sample when detected with phosphospecific  $\alpha$ L antibody. We conclude that a maximum of 40% of  $\alpha$ L is constitutively phosphorylated in T cells.

#### Mutation of Ser1140 of $\alpha$ L abolishes T cell adhesion to ICAM-1 induced by the activating antibody MEM-83 and MEM-83-induced affinity regulation of LFA-1

We next generated stable J- $\beta$ 2.7 cell transfectants expressing wt  $\alpha$ L and S1140A- $\alpha$ L to examine the role of the phosphorylation site in cells (Fig. 3 A). Mutation of Ser1140 to Ala did not affect the heterodimerization or cell surface expression of LFA-1. The phosphospecific  $\alpha$ L antibody was used for the characterization of the J- $\beta$ 2.7 cell clones. The  $\alpha$ L chain was easily detected in J- $\beta$ 2.7 wt  $\alpha$ L cells, whereas no significant staining of  $\alpha$ L was seen in J- $\beta$ 2.7 S1140A- $\alpha$ L cells with or without phorbol ester activation (Fig. 3 B).

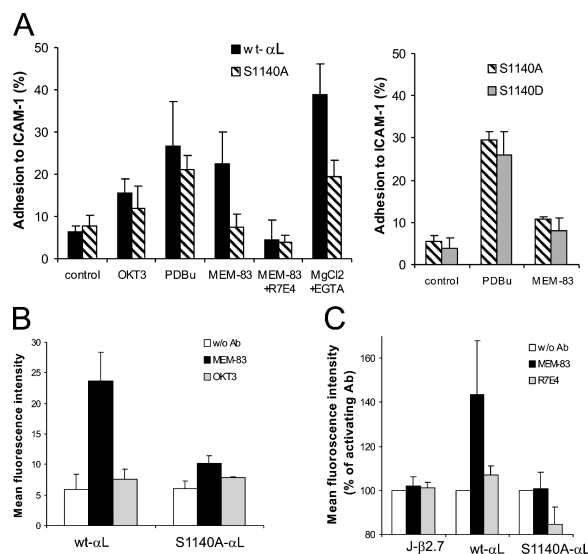
We went on to study the roles of the  $\alpha$ L phosphorylation site in cells that were stimulated with different agonists. The binding of both wt  $\alpha$ L and S1140A- $\alpha$ L transfectants to the immobilized ligand intercellular adhesion molecule (ICAM)-1 was increased by TCR ligation and by phorbol ester treatment, as compared with resting cells (Fig. 4 A). In contrast, cells expressing S1140A- $\alpha$ L bound less efficiently to ICAM-1 when activated by MEM-83. It has been speculated that MEM-83 activates LFA-1 binding to ICAM-1 by changing the orientation of the I domain in the  $\alpha$ L, which leads to conformational change at the ICAM-1 binding site (Lu et al., 2004). Mg/EGTA also induces a conformational change, probably at the MIDAS and ADMIDAS sites of the I-like domain of the  $\beta$ 2 subunit (Shimaoka et al., 2002). Mg/EGTA increased the  $\alpha$ L-mutated cell adhesion to ICAM-1, but not as effectively as in wt cells (Fig. 4 A). These results indicate that  $\alpha$ L phosphorylation

could be involved in LFA-1 conformational changes needed for integrin binding to ligand, but not in integrin activation induced by TCR ligation and phorbol ester stimulation. The latter stimuli lead to increased binding of cells to coated ICAM-1, but previous studies have reported that they did not induce conformational changes in the integrin (Cabanas and Hogg, 1993;



**Figure 3. Mutation of the Ser1140  $\alpha$ L phosphorylation site in J- $\beta$ 2.7 transfectants.** (A) Surface expression of integrin LFA-1  $\beta$ 2 chains (R7E4 antibody staining) and wt and S1140A- $\alpha$ L chains (MEM-83 antibody staining) in J- $\beta$ 2.7 wt cells and J- $\beta$ 2.7 wt  $\alpha$ L and J- $\beta$ 2.7 S1140A- $\alpha$ L transfectants was examined by flow cytometry. As a control, the antibody OKT3 was used to detect similar amounts of CD3 in all clones. Dotted lines represent the background fluorescence and solid lines the specific antibody labeling. (B) LFA-1 in S1140A- $\alpha$ L-expressing J- $\beta$ 2.7 cells cannot be phosphorylated on Ser1140, as detected by the phosphospecific antibody against  $\alpha$ L.  $\beta$ 2 was detected with a blotting antibody to demonstrate equal loading of LFA-1.





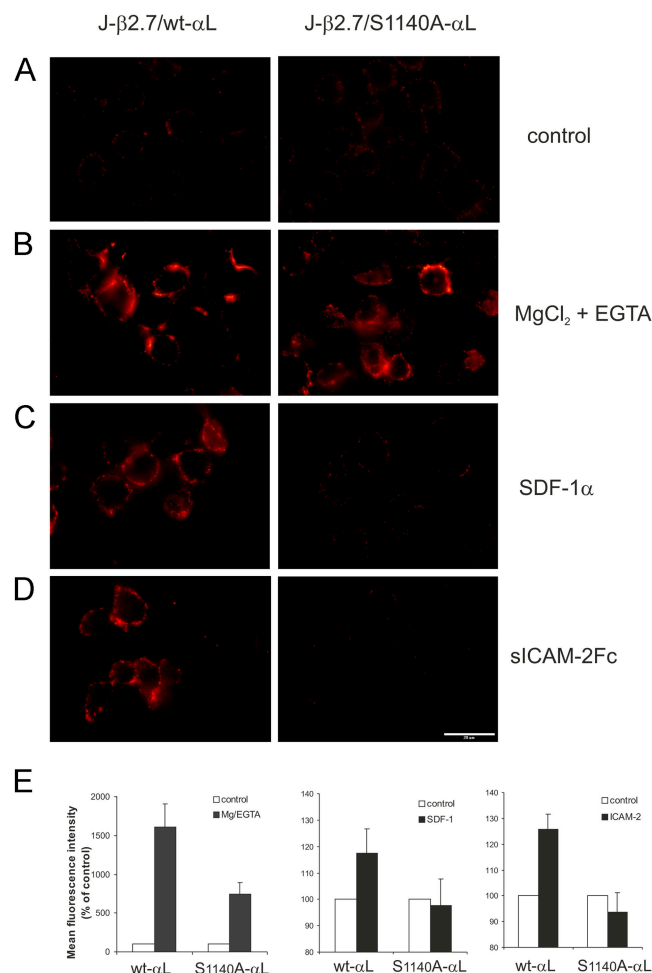
**Figure 4. Effect of mutation of the phosphorylation site in the LFA-1  $\alpha$ L chain on adhesion to ICAM-1 and induction of high-affinity LFA-1.** (A) Mutation of  $\alpha$ L chain Ser1140 affects adhesion induced by an activating antibody to LFA-1. J- $\beta$ 2.7 transfectants expressing wt  $\alpha$ L or S1140A- $\alpha$ L were allowed to bind to ICAM-1-coated wells without stimulation (control) or after stimulation with 10  $\mu$ g/ml OKT3, 200 nM PDBu, 5 mM  $MgCl_2$ /1 mM EGTA, or 10  $\mu$ g/ml MEM-83 in the presence or absence of the  $\beta$ 2-blocking antibody R7E4. (right) Adhesion of S1140A- $\alpha$ L and S1140D- $\alpha$ L-transfected cells to ICAM-1 after phorbol ester or MEM-83 stimulation was compared. (B) Effects of the S1140A mutation on sICAM-1-binding activity of LFA-1. J- $\beta$ 2.7 transfectants were activated with 10  $\mu$ g/ml MEM-83 or control antibody OKT3 in the presence of 150  $\mu$ g/ml ICAM-1Fc and binding was determined by flow cytometry. (C) The effects of the S1140A mutation on expression of the  $\alpha$ L activation reporter mAb24 were examined. J- $\beta$ 2.7 cells were incubated with mAb24 in the presence of MEM-83 or R7E4, and mAb24 expression was analyzed by flow cytometry. Error bars represent SD.

Stewart et al., 1996). Whether a negative charge at position 1140 would be enough to affect adhesion was studied by using a Ser1140Asp mutation (Fig. 4 A, right). The result showed that the S/A and S/D mutations worked similarly.

To further study the mechanisms of the different modes of adhesion, a soluble ICAM-Fc (sICAM-1Fc) binding assay was used to measure the ligand-binding activity. mAb MEM-83 greatly increased the binding of sICAM-1 to wt  $\alpha$ L transfectants (Fig. 4 B), whereas TCR stimulation had no effect. Importantly, only minimal sICAM-1 binding was observed for S1140A- $\alpha$ L cells after MEM-83 activation. Affinity modulation of LFA-1 can also be detected using mAb24 (Dransfield and Hogg, 1989). This epitope was not expressed in resting J- $\beta$ 2.7 transfectants, but there was enhanced expression on the wt cells after MEM-83 activation (Fig. 4 C). The S1140A mutation abolished MEM-83-induced expression of the mAb24 epitope (Fig. 4 C). Together, the J- $\beta$ 2.7 cells expressing nonphosphorylatable S1140A- $\alpha$ L mutant show a marked reduction in ICAM-1 binding and expression of the high-affinity form of integrin.

#### The phosphorylation site in $\alpha$ L is involved in chemokine- and ligand-induced affinity regulation of the integrin

One of the best characterized examples of LFA-1 affinity modulation is the activation of integrin-dependent leukocyte arrest



**Figure 5. The Ser1140Ala mutation inhibits mAb24 expression induced by SDF-1 or ICAM-2.** (A) J- $\beta$ 2.7 cells expressing wt  $\alpha$ L or S1140A- $\alpha$ L. (B) Cells were activated with 5 mM  $MgCl_2$ /1 mM EGTA for 30 min. (C) Cells were activated with 3  $\mu$ g/ml SDF-1 $\alpha$  for 2 min; (D) Cells were activated with 10  $\mu$ g/ml sICAM-2Fc for 30 min, and the high-affinity epitope of LFA-1 was examined by fluorescence microscopy with mAb24. Bar, 20  $\mu$ m. (E) mAb24 expression was quantified using flow cytometry. Error bars represent SD.

and migration through G protein-coupled receptors for chemokines (Constantin et al., 2000). SDF-1 $\alpha$ , a CXC chemokine, has been shown to induce a rapid and transient activation of LFA-1 in J- $\beta$ 2.7 wt  $\alpha$ L cells that can be detected by expression of the mAb24 epitope (Weber et al., 1999). To determine whether the S1140A mutation affected chemokine-triggered mAb24 expression, we stimulated J- $\beta$ 2.7 transfectants with SDF-1 $\alpha$  and studied the mAb24 epitope by immunofluorescence. No mAb24 staining was seen in nonstimulated cells (Fig. 5 A). Mg/EGTA treatment was used as a positive control to show the ability of transfectants to express the mAb24 epitope (Fig. 5, B and E). After SDF-1 $\alpha$  stimulation, clear staining of mAb24 was detected on the wt  $\alpha$ L cells, but not on the S1140A- $\alpha$ L-expressing cells (Fig. 5, C and E).

Furthermore, integrin ligands are known to increase adhesion, probably by affinity modulation (Cabanas and Hogg, 1993; Kotovvuri et al., 1999). sICAM-2 increased the expression of the mAb24 epitope on wt  $\alpha$ L cells, whereas no staining of the S1140A- $\alpha$ L cells was observed (Fig. 5, D and E). Thus, physio-

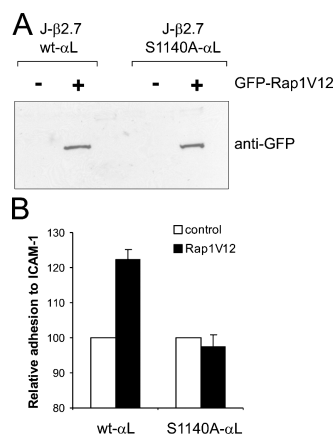


Figure 6. **Rap1V12 is unable to activate the Ser1140A-mutated integrin in cells.** (A) The expression levels of GFP-Rap1V12 in J-β2.7 cell transfectants is shown. (B) Adhesion to coated ICAM-1 of the transfectants was measured as described in Materials and methods. Error bars represent SD.

logical activators of the LFA-1 integrin, which have been shown to lead to extracellular conformational changes, are not able to activate integrins with nonphosphorylatable αL chains.

#### Active Rap1 cannot activate the S1140A-αL-mutated integrin in cells

The small GTPase Rap1 is a potent activator of LFA-1 that is needed in different modes of LFA-1 activation, including activation from the outside of the cell with divalent cations or antibodies (Katagiri et al., 2000, 2002; de Bruyn et al., 2002; Sebzda et al., 2002). Transfection of leukocytes with an active form of Rap1 (Rap1V12) has been shown to increase LFA-1 affinity, as measured by increased ability to bind sICAM-1 (Katagiri et al., 2003). GFP-Rap1V12 was transfected into J-β2.7

cells stably expressing wt αL or S1140A-αL (Fig. 6). The expression level of Rap1V12 in wt αL- and S1140A-αL-expressing J-β2.7 cells was the same (Fig. 6 A). However, Rap1V12 was unable to activate the S1140A mutant, even if increased adhesion of wt αL-expressing cells could be observed after Rap1V12 transfection (Fig. 6 B).

#### Thr758-phosphorylated β2 binds 14-3-3 in vitro and in vivo

Having established the phosphorylation site in αL and its functional characteristics, we focused on the investigation of the β2 polypeptide phosphorylation. Both phorbol ester activation and TCR ligation induce phosphorylation of the β chain on Thr758 (Hilden et al., 2003), which is the first threonine in the TTT motif that is important for adhesion and cytoskeletal interactions of the LFA-1 and other integrins (Fig. 7 A; Hibbs et al., 1991; Peter and O'Toole, 1995; Fagerholm et al., 2004). 14-3-3 proteins from leukocyte lysates have previously been shown to interact with a synthetic β2 integrin peptide phosphorylated on Thr758 (Fagerholm et al., 2002b), but whether the binding was direct or indirect and whether it occurred in cells was not established. In this study, we examined the binding of purified 14-3-3 proteins to the phosphorylated integrin cytoplasmic peptides. The proteins bound specifically to the phosphorylated, but not to the nonphosphorylated, β2 chain, as examined by peptide affinity chromatography with nonphosphorylated and Thr758-phosphorylated β2 chain peptides (Fig. 7 B). 14-3-3 proteins could be eluted from the affinity column by the peptide ARAApSAPA, which specifically binds to the phosphopeptide-binding groove in 14-3-3 (Moorhead et al., 1999), but not by a control peptide (phospho-αL peptide; Fig. 7 B). This shows that the binding is specific and occurs through the canonical 14-3-3 phosphopeptide binding motif (Fig. 7 B). Phosphorylation

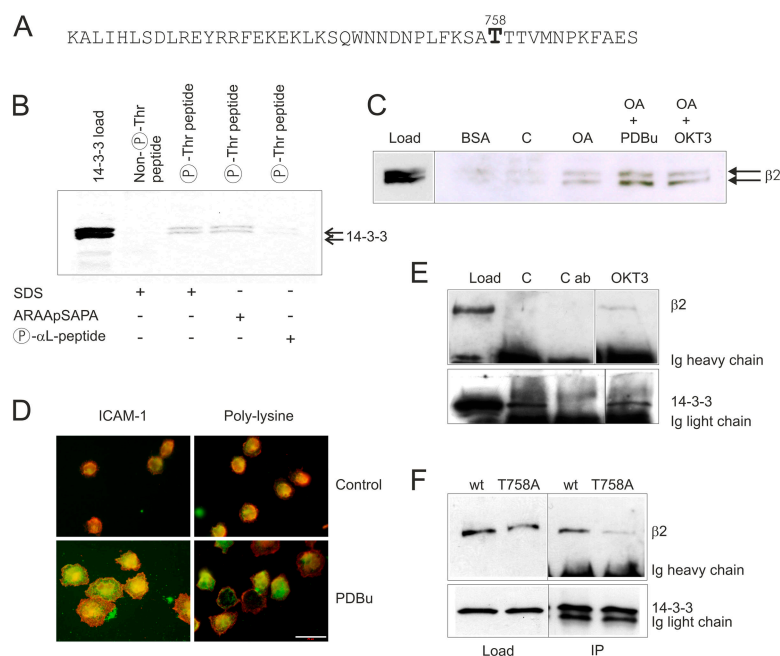


Figure 7. **Specific binding of 14-3-3 proteins to Thr758-phosphorylated β2 integrin in vitro and in vivo.** (A) Sequence of the β2 integrin cytoplasmic domain with the Thr758 phosphorylation site shown in bold. (B) Binding of purified yeast 14-3-3 proteins to Thr758-phosphorylated and nonphosphorylated peptides was assessed by peptide affinity chromatography and Coomassie staining of eluted proteins. Elution was performed with SDS, the ARAAPsAPA peptide, or a control peptide (phospho-αL peptide). (C) Binding of β2 integrin from control (C), okadaic acid-pretreated (OA), okadaic acid-pretreated and phorbol ester-activated (OA + PDBu), or TCR-ligated (OA + OKT3) Jurkat cell lysates to 14-3-3-Sepharose or BSA columns. The eluted proteins were detected by Western blotting with β2 integrin antibodies. The two bands detected in the loading control and stimulated cells presumably represent precursor and mature forms of the integrin β chain found in Jurkat cells (Fagerholm et al., 2002a). (D) Coimmunofluorescence of 14-3-3 (green) and β2 integrins (red) in untreated (control) human T cells or in cells activated with phorbol ester (PDBu) on poly-lysine-coated coverslips. Bar, 20 μm. (E) Increased binding between 14-3-3 and β2 in TCR-ligated cells. Endogenous 14-3-3 proteins were immunoprecipitated from human T cell lysates with a control antibody (C ab) or a 14-3-3 mAb, and coimmunoprecipitating β2 integrins were detected by Western blotting with β2 integrin antibodies. Immunoprecipitated 14-3-3 proteins were detected with a 14-3-3 blotting antibody to demonstrate equal loading. (F) wt β2 or T758A-β2 transfected COS-1 cell lysates were used in the immunoprecipitation performed as in E.

of another major site in the  $\beta 2$  cytoplasmic domain, Ser756, has previously been shown not to mediate 14-3-3 binding (Fagerholm et al., 2002b).

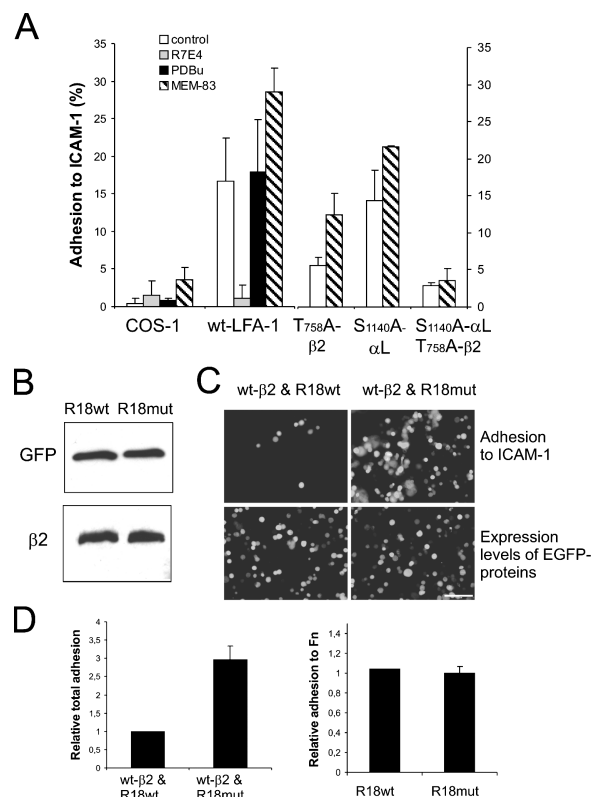
Affinity chromatography with 14-3-3 proteins coupled to Sepharose showed specific interaction between 14-3-3 and  $\beta 2$  integrins, which occurred only when cells had been activated with phorbol ester or TCR ligation (Fig. 7 C). Ligand binding has been shown to cluster integrins on the cell surface (Kim et al., 2004). Indeed, in coimmunofluorescence studies, the 14-3-3 proteins and  $\beta 2$  integrins coclustered in activated T cells spread on ICAM-1, as shown by the increased yellow staining in these cells compared with cells on poly-lysine or nonstimulated cells (Fig. 7 D). Importantly, endogenous 14-3-3 proteins and  $\beta 2$  integrins could be coprecipitated from TCR-stimulated, but not unstimulated, human T cells (Fig. 7 E). To confirm the Thr758-dependent association between 14-3-3 and  $\beta 2$ , we transfected COS-1 cells with wt  $\beta 2$  or T758A-mutated  $\beta 2$  and performed coprecipitation experiments with endogenous 14-3-3 proteins. The transfected Thr758Ala-mutated integrin showed reduced association with endogenous 14-3-3 proteins in most coimmunoprecipitation experiments (Fig. 7 F).

#### Mutation of Thr758 in $\beta 2$ or blocking of 14-3-3 binding to the $\beta 2$ chain reduces constitutive COS cell adhesion

Mutation of Thr758 in the  $\beta 2$  chain has previously been shown to affect adhesion of LFA-1 to ICAM-1 when transfected into COS cells, where LFA-1 is constitutively active, and into LAD cells (a B lymphoblastoid cell line lacking LFA-1), where LFA-1 can be activated with phorbol ester (Hibbs et al., 1991). We used the COS cell system to investigate the effect of direct 14-3-3 association with the  $\beta 2$  chain without interfering with activating signals of the integrin and because a T cell model lacking  $\beta 2$  integrins is currently unavailable.

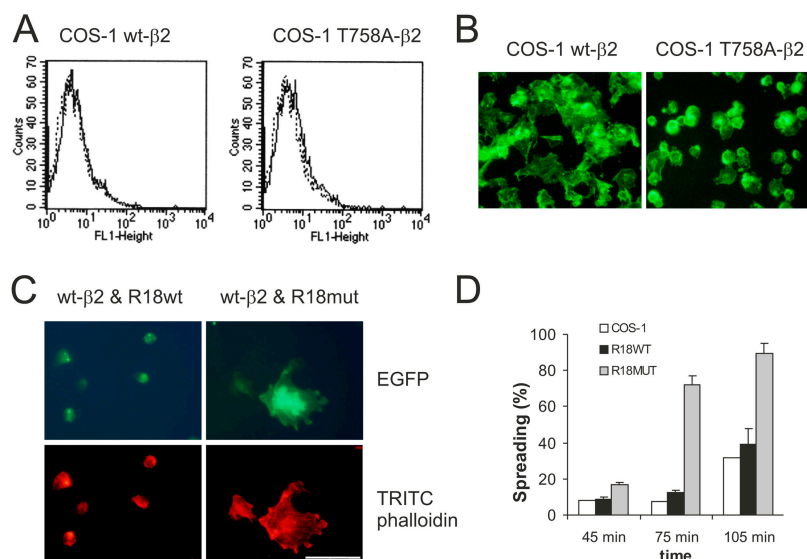
Phorbol ester did not stimulate LFA-1-mediated adhesion to ICAM-1 in this system, but MEM-83 did have a stimulatory effect (Fig. 8 A). As previously reported, the Thr758Ala mutation significantly reduced the constitutive integrin-mediated adhesion of transfected cells to ICAM-1 (Fig. 8 A). MEM-83 could still activate the Thr-mutated integrin, albeit to a lower degree than for wt LFA-1 (Fig. 8 A), showing that activating conformational changes could still occur for the mutated integrin. However, when both Ser1140 and Thr758 were mutated, not even MEM-83 could activate the integrin-mediated adhesion (Fig. 8 A), even if the singly mutated Ser1140Ala- $\alpha L$  together with wt  $\beta 2$  could normally mediate adhesion in COS cells. Because the S1140A- $\alpha L$ -mutated integrin is adhesion deficient in J- $\beta 2.7$  cells, but not in COS-1 cells, it is clear that these cells are not a useful model for studying LFA-1 function in all cases.

To examine whether the effects of the Thr758 mutation seen were attributable to the blockage of 14-3-3 proteins from binding to the integrin, we cotransfected cells with wt LFA-1 integrins and an EGFP-R18wt construct, which blocks 14-3-3 interactions with its cellular ligands by binding to the phosphopeptide-binding groove in 14-3-3 (Jin et al., 2004). As a



**Figure 8. Effect of the mutation of Thr758 or blocking 14-3-3 binding to the  $\beta 2$  chain on adhesion to ICAM-1.** (A) Mutation of Thr758 of  $\beta 2$  reduces COS cell adhesion to ICAM-1. COS-1 cells were transfected with wt  $\alpha L$  or S1140A- $\alpha L$  and wt  $\beta 2$  or Thr758/Ala-mutated  $\beta 2$  integrin cDNA, and cell adhesion to coated ICAM-1 in the presence of PDBu, blocking antibody (R7E4), or stimulating antibody (MEM-83) was examined. (B) Cells were cotransfected with wt LFA-1 integrins and EGFP-R18wt or EGFP-R18mut constructs. Levels of EGFP proteins and LFA-1 in the transfected cells were examined by Western blotting. (C) Adhesion of COS cells was visualized by immunofluorescence of EGFP-expressing cells adhering to ICAM-1-coated plates (top). Bar, 100  $\mu m$ . (bottom) EGFP levels in the transfected cells. (D, left) Total cells adhering to ICAM-1 were also quantified as described in Materials and Methods. (D, right) As a control, the R18wt- and R18mut-transfected cells were also examined for adhesion of endogenous COS cell integrins to fibronectin. Error bars represent SD.

control, we used a mutant construct that does not bind to 14-3-3 proteins (R18mut). The level of GFP proteins and cell surface LFA-1 integrins in the EGFP-positive cells was equal in R18wt and R18mut cells, as examined by flow cytometry (unpublished data), Western blotting with an anti-GFP antibody and an LFA-1 blotting antibody (Fig. 8 B), and immunofluorescence (Fig. 8 C). R18wt cotransfected cells showed markedly reduced adherence to ICAM-1 (Fig. 8, C and D). The 14-3-3 proteins have many binding partners among cytoskeletal and signaling proteins that might affect adhesion, and R18 peptides have indeed been shown to alter the cytoskeleton in cells that do not express LFA-1 (Jin et al., 2004). However, when the adhesion of endogenous COS cell integrins to fibronectin was examined there was no difference in adhesion between R18wt and R18mut-transfected cells (Fig. 8 D, right). Thus, it is likely that 14-3-3 binding to the Thr758-phosphorylated integrin mediates the effect of Thr758 on cell adhesion.



**Figure 9. Blocking the interaction between 14-3-3 and  $\beta 2$  integrins abolishes actin reorganization and cell spreading.** (A) COS cells were transfected with wt  $\beta 2$  or T758A- $\beta 2$ , and the transfected COS cells were examined for mAb24 expression by flow cytometry as in Fig. 3. Dotted lines represent background fluorescence and solid lines represent the mAb24 binding. (B) Cells were allowed to spread on coated ICAM-1. The cells were examined for F-actin by FITC-phalloidin staining. (C) R18wt or R18mut was cotransfected with wt LFA-1 into COS-cells, and cell spreading on ICAM-1 was visualized. Both EGFP (R18) expression and TRITC-phalloidin staining is shown from the same cells. Bar, 50  $\mu$ m. (D) Spreading was quantified by counting the percentage of spread transfected cells at different time points. Error bars represent SD.

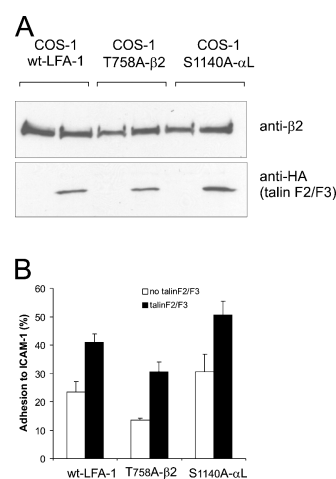
### 14-3-3 binding to phosphorylated $\beta 2$ is involved in cytoskeletal rearrangements

Neither the wt LFA-1 nor the Thr758Ala mutant of the  $\beta 2$  chain showed any binding of mAb24 in COS cells (Fig. 9 A). Additionally, phorbol esters and TCR ligation have been reported not to induce sICAM-1 binding or mAb24 expression in T cells, although cell adhesion to coated ICAM-1 can be readily detected (Stewart et al., 1996). In contrast, TCR triggering and phorbol esters have been closely associated with a spreading phenotype of T cells (Stewart et al., 1996). Furthermore, the TTT motif of the integrin has been closely associated with actin reorganization events, but not affinity changes, in integrins (Peter and O'Toole, 1995). Indeed, the Thr758 mutation was shown to significantly reduce cell spreading on ICAM-1 as examined by FITC-phalloidin staining of polymerized actin (Fig. 9 B). In addition, R18wt-transfected cell spreading on ICAM-1 was almost completely abolished (Fig. 9, C and D). Quantitation of transfected cell spreading showed that R18wt and LFA-1 cotransfected cells only spread at later time points, when other untransfected COS-1 cells started spreading unspecifically on ICAM-1 (Fig. 9 D). The mutant R18 construct did not block spreading. These results show that 14-3-3 binding to the phosphorylated  $\beta 2$  integrin is important for integrin effects on the cell cytoskeleton.

### A talin head domain construct can activate the Thr758Ala-mutated $\beta 2$ integrin in cells

The head domain of the large cytoskeletal protein talin has been demonstrated to be a major player in activation of different integrins, including LFA-1 (Kim et al., 2003; Tadokoro et al., 2003). It binds directly to the  $\beta$  chains of integrin cytoplasmic domains and, presumably, induces a separation of the cytoplasmic domains of the  $\alpha$  and  $\beta$  chains, which leads to a conformational change in the extracellular domain (Kim et al., 2003; Tadokoro et al., 2003). Thus, we examined whether the effect of the T/A mutation of the  $\beta 2$  chain was attributable to an altered ability of talin to bind to and activate LFA-1.

We cotransfected COS cells with wt or T758A-mutated  $\beta 2$  together with the F2/F3 domains of the talin head domain. The expression levels of LFA-1 and the talin head domain construct were similar in the different transfectants, as measured by Western blotting (Fig. 10 A) and flow cytometry (unpublished data). We saw an increase in LFA-1-mediated adhesion to ICAM-1 in the talin head cotransfected cells as compared with cells without the talin head (Fig. 10 B), although the occurrence of adhesion was somewhat less in T758A- $\beta 2$ -transfected cells than in the wt controls. The talin head domain, like the antibody MEM-83, could activate S1140A-mutated  $\alpha L$  in the COS cell system (Fig. 10 B). Thus, in COS-1 cells, affinity-inducing stimuli of the integrin are also functional for S1140A-mutated  $\alpha L$ , indicating the possibility that a leukocyte-specific restrictive factor working through the  $\alpha$  chain is missing.



**Figure 10. The head domain of talin can activate the Thr758Ala-mutated  $\beta 2$  integrin.** (A) COS cells were transfected with wt  $\beta 2$  or T758A- $\beta 2$  or S1140A- $\alpha L$  mutant constructs, together with HA-tagged talin F2/F3 domains, and the level of protein expression was examined by Western blotting with an LFA-1 binding antibody or an anti-HA antibody. (B) COS cell adhesion of the transfected cells to coated ICAM-1 was examined as described in Materials and methods. Error bars represent SD.



## Discussion

Phosphorylation of the cytoplasmic domains of LFA-1 was initially reported more than a decade ago (for review see Fagerholm et al., 2004), but the significance of these phosphorylation events in integrin regulation has remained unclear. Threonine phosphorylation of  $\beta 2$  has been reported (Valmu and Gahmberg, 1995; Hilden et al., 2003), and it has also been shown that the threonine triplet in  $\beta 2$  (Thr 758–760) is important for both adhesion and cytoskeletal reorganization mediated by LFA-1 (Hibbs et al., 1991; Peter and O'Toole, 1995). The stoichiometry of the phosphorylation of  $\beta 2$  integrin in phorbol ester-stimulated T cells in the presence of okadaic acid was determined to be 0.92 mol/mol of protein (Valmu et al., 1999b). Because several sites become phosphorylated under these conditions it is clear that each site is phosphorylated at a relatively low level (Fagerholm et al., 2002b; Hilden et al., 2003). However, in comparison with receptor tyrosine phosphorylation, low stoichiometry phosphorylations may still play important biological roles by regulating protein–protein interactions.

$\alpha L$  has been shown to be constitutively phosphorylated in T cells (Hara and Fu, 1986; Chatila et al., 1989), but the phosphorylation site and stoichiometry of  $\alpha L$  phosphorylation has not been assessed. We have now mapped the phosphorylation site in the  $\alpha L$  cytoplasmic domain to Ser1140 and shown that  $\sim 40\%$  of surface  $\alpha L$  was phosphorylated in T cells. A similarly high stoichiometry of phosphorylation already found in resting cells has been shown for the  $\alpha 4$  integrin (Han et al., 2001). In analogy with the  $\alpha 4$  integrin, where paxillin binding to the nonphosphorylated integrin rather than to the phosphorylated form excludes this adaptor protein from the leading edge of the cell and thus regulates cell migration (Goldfinger et al., 2003), constitutive phosphorylation of the  $\alpha L$  tail may play profound roles in spatiotemporal regulation of integrin functions.

By mutating Ser1140, we have shown that the nonphosphorylatable LFA-1  $\alpha$  chain expressed in a T cell line can no longer be activated by agents that have previously been demonstrated to induce high-affinity forms of the integrin; i.e., by an activating antibody (MEM-83); by ligands or chemokines, as detected by adhesion to ICAM-1; by sICAM-1 binding; or by mAb24 that detects the high-affinity form of LFA-1. Thus, phosphorylated Ser1140 is involved in conformational changes occurring in LFA-1 in response to several different affinity-increasing stimuli. One possibility is that the negative charge induced by phosphorylation could facilitate the separation of the integrin cytoplasmic tails, leading to a conformational change in the extracellular domain (Kim et al., 2004; Adair et al., 2005). However, a Ser-Asp mutation did not lead to a different adhesion phenotype compared with the Ser-Ala mutation, indicating that simply substituting a negatively charged amino acid for serine is not enough to mimic phosphorylation. Thus, it is more plausible that the  $\alpha L$  phosphorylation site works through selective binding to some cytoplasmic factor.

The small GTPase Rap1 is an important mediator of various modes of LFA-1 activation in leukocytes, including activation by antibodies and divalent cations (Katagiri et al., 2000, 2002; de Bruyn et al., 2002; Sebzdka et al., 2002). Importantly,

Rap1 has been shown to act through the integrin  $\alpha$  chain cytoplasmic domain and to influence integrin affinity for ICAM-1 (Tohyama et al., 2003). Thus, we wanted to examine the effect of active Rap1 (Rap1V12) on the nonphosphorylatable S1140A mutant. Rap1V12 was able to induce binding of wt  $\alpha L$ -transfected, but not mutant- $\alpha L$ -transfected, J- $\beta 2.7$  cells to coated ICAM-1. The mutated  $\alpha L$  may be locked in a low-affinity conformation that cannot be activated from outside of the cell by activating antibodies or other activating stimuli that increase integrin affinity for its ligands. However, it can still be activated with phorbol ester and TCR stimulation, which do not (and, if so, only marginally) influence integrin affinity.

Rap1 may be working upstream of the  $\alpha L$  phosphorylation, possibly influencing the binding of some cytoplasmic factor to the  $\alpha L$  cytoplasmic tail. RapL is a Rap1-binding molecule that can activate LFA-1 and is found in leukocytes (Katagiri et al., 2003). It will be of future interest to study the effect of RapL in this system.

It has previously been reported that the 14-3-3 isoforms  $\alpha\beta$  and  $\delta\zeta$  from leukocyte lysates associate specifically with a Thr758-phosphorylated  $\beta 2$  integrin COOH-terminal peptide (Fagerholm et al., 2002b). We discovered that the  $\beta 2$ –14-3-3 interaction is direct. Additionally, we have shown that the binding occurs in T cells that have been activated by stimuli that induce phosphorylation of the  $\beta 2$  chain on Thr758, i.e., phorbol ester and TCR engagement. Mutation of Thr758 or blocking of 14-3-3 binding to the  $\beta 2$  integrin by R18 peptides inhibited cell adhesion to ICAM-1. The Thr758 mutation or R18 peptide also inhibited integrin-mediated cell spreading on ICAM-1. Thus, phosphorylation of Thr758, which is induced by inside-out activating stimuli for the integrin, mediates binding to 14-3-3 proteins in cells, and this interaction seems to mediate the effect of the integrin on the cell cytoskeleton. The 14-3-3 proteins are dimers, and both monomers can independently bind to phosphorylated targets either within the same protein or in different proteins (Tzivion and Avruch, 2002; MacKintosh, 2004). It is possible that threonine phosphorylation of CD18 recruits 14-3-3 proteins to the plasma membrane–cytoskeleton connection and that the 14-3-3s in turn recruit other proteins to regulate downstream events.

There are increasing amounts of evidence that talin plays a profound role in activating several integrins, including LFA-1 (Kim et al., 2003; Calderwood, 2004; Smith et al., 2005). The head domain of talin binds to integrin  $\beta$  chains and induces a separation of  $\alpha$  and  $\beta$  chain cytoplasmic domains. Therefore, we investigated the ability of the talin head domain construct to activate the  $\beta 2$ -T758A mutant. It could still be activated by the talin construct in cells, as measured by increased adhesion of the transfected cells to coated ICAM-1. The talin-binding site in  $\beta 3$  integrins encompasses the first NPXY motif (Tadokoro et al., 2003) and corresponds to residues 747–755 in  $\beta 2$ , which precede Thr758. Thus, the mechanism by which Thr758 regulates adhesion is presumably not related to talin.

The cytoskeletal protein  $\alpha$ -actinin has been shown to associate with the  $\beta 2$  tail at a membrane-proximal site, whereas the COOH-terminal portion of the tail (residues 748–762) inhibits this interaction (Sampath et al., 1998). Substitution of

Thr758 with Ala or with the phosphate-mimic Glu stimulates binding of  $\alpha$ -actinin to  $\beta 2$ , suggesting that phosphorylation of  $\beta 2$  on Thr758 does not regulate  $\alpha$ -actinin binding (Sampath et al., 1998). Additionally, deletion of the  $\alpha$ -actinin binding site in  $\beta 2$  does not influence adhesion to ICAM-1. On the other hand, other proteins may bind to the TTT region and, indeed, filamin has been shown to do so (Calderwood et al., 2001). Thus, it is possible that threonine phosphorylation and 14-3-3 binding may regulate such interactions.

LFA-1 is involved in many different immunological adhesion events. It can be activated by different stimuli and is regulated both by conformational changes and by cytoskeletal attachment and clustering. It is now clear that phosphorylation of both the  $\alpha$  and  $\beta$  chains plays a role in the molecular mechanisms involved in the different activation events. However, the  $\alpha L$  and  $\beta 2$  polypeptides play distinctive roles in integrin activation. The integrin  $\alpha L$  chain is constitutively phosphorylated, and this phosphorylation site (Ser1140) seems to be required for adhesion events that involve rapid changes in the conformation and affinity of the integrin heterodimer. In contrast, the  $\beta 2$ -Thr758 phosphorylation that is induced after physiological triggering of T cells through the TCR works through interaction with 14-3-3 proteins. This form of adhesion is slower and involves actin reorganization and cell spreading. The contribution of the different phosphorylation events to overall adhesion may depend on the context of adhesion and provides the possibility for regulation of both fast, transient adhesive events and long-term, stable adhesion strengthening.

## Materials and methods

### Reagents

Phorbol 12,13-dibutyrate (PDBu) was obtained from Sigma-Aldrich. [ $^{32}$ P]orthophosphate (aqueous solution, 10 mCi/ml, 5,000 Ci/mmol) was purchased from the Radiochemical Centre (GE Healthcare). Recombinant human ICAM-1, ICAM-1Fc, and ICAM-2Fc were obtained from R&D Systems, and fibronectin was obtained from Calbiochem. Sequencing-grade modified trypsin (activity more than 2.5 U/mg) was purchased from Promega. G418 was purchased from Calbiochem-Novabiochem. SDF-1 was obtained from R&D Systems. The peptides (ARAAPSA, CLFKSATTVMN, CLFKSApTTVMN, and CLKPLHEKDSEpSGGGKD, where pS is phosphoserine and pT is phosphothreonine) were synthesized by Fmoc chemistry (Valmu et al., 1999b).

### Antibodies

The mAbs R7E4 and R2E7B against the human  $\beta 2$  subunit of leukocyte integrin have been described previously (Nortamo et al., 1988). The monoclonal activating antibody against CD3, OKT3, was purified from ascites fluid produced by hybridoma cells (clone CRL 8001; American Type Culture Collection). The activating mAb MEM-83 was provided by V. Horejsi (Institute of Molecular Genetics, Prague, Czech Republic). IB4 was a gift from M.A. Arnaout (Massachusetts General Hospital, Boston, MA). mAb24 ( $\beta 2$ ) was a gift from N. Hogg (Imperial Cancer Research Fund, London, UK). The 14-3-3 antibodies H-8 and K-19, as well as the GFP antibody, were obtained from Santa Cruz Biotechnology, Inc. The HA-tagged talin F2/F3 protein was detected by anti-HA-biotin antibodies (Roche) and streptavidin-HRP (GE Healthcare). To produce antiserum to phospho- $\alpha L$ , the integrin  $\alpha L$  chain phosphopeptide CLKPLHEKDSEpSGGGKD was conjugated to keyhole limpet hemocyanin (Harlow and Lane, 1988). The complex was injected into rabbits. The antisera were purified by affinity chromatography using the phosphorylated peptide.

### cDNA constructs

The EGFP-R18 and EGFP-R18mut plasmids were gifts from T. Pawson (University of Toronto, Toronto, Canada). The 14-3-3 *Saccharomyces*

*cerevisiae* isoforms BMH1 and BMH2 plasmids in DH5 $\alpha$  were gifts from C. MacKintosh (University of Dundee, Nethergate, Dundee, Scotland, UK). The HA-tagged talin F2/F3 construct was a gift from D. Calderwood (Yale University, New Haven, CT). GFP-Rap1V12 was a gift from D. Cantrell (University of Dundee). cDNA coding for full-length human  $\alpha L$  was subcloned into the pcDNA3 vector and human  $\beta 2$  was subcloned into the  $\pi$ HM3 vector. Mutants were created using site-directed mutagenesis (Weiner et al., 1994), and the mutated constructs were checked by sequencing.

### Cell lines and transfection

Buffy coats used for the isolation of T cells were obtained from the Finnish Red Cross Blood Transfusion Service (Valmu et al., 1999b). The cells were grown in RPMI 1640 medium supplemented with 10% FCS, L-glutamine, and antibiotics. The Jurkat cell clone E6.1 (American Type Culture Collection) was maintained in the same medium.

COS-1 cells were cultured in DME supplemented with 10% FCS, L-glutamine, and antibiotics. COS-1 cells were used for transient expression of wt and mutant  $\beta 2$  integrins. COS-1 cells were cotransfected with purified  $\alpha$ - and  $\beta$ -subunit cDNAs with or without EGFP-R18wt, EGFP-R18mut, or talin F2/F3 constructs using the FuGENE 6 transfection reagent according to the manufacturer's instructions (Roche). Flow cytometric analysis was used to quantify cell surface expression of integrins in the transfected COS-1 cells.

The human T lymphoma cell line clone J- $\beta 2.7$ , which lacks the LFA-1  $\alpha$  chains and was derived from Jurkat cells by mutagenesis (Weber et al., 1997), was a gift from N. Hogg. J- $\beta 2.7$  cells ( $7.2 \times 10^6$  per transfection) were washed, suspended in 0.36 ml PBS, and mixed with 20  $\mu$ g wt  $\alpha L$  or S1140A- $\alpha L$  DNA. Electroporation was performed at 240 V and 950  $\mu$ F. After 48 h of culture, the medium was supplemented with 0.8 mg/ml G418. LFA-1-expressing cells were enriched using magnetic cell sorting (Miltenyi Biotec GmbH). Alternatively, cells (S1140D- $\alpha L$  and Rap1V12) were transfected with the Optifect system according to the manufacturer's instructions (Invitrogen). Flow cytometric analysis was used to quantify cell surface expression of integrins in the transfected cells. For both wt  $\alpha L$ - and S1140A- $\alpha L$ -expressing J- $\beta 2.7$  cells, several clones were selected that exhibited comparable levels of surface expression as detected by flow cytometry. In each experiment, we used two independent clones of both wt and  $\alpha L$  mutant cells.

### Peptide affinity chromatography

The peptides CLFKSATTVMN and CLFKSApTTVMN, corresponding to the  $\beta 2$  integrin sequence surrounding the phosphorylated Thr758, were coupled to thiopropyl-Sepharose according to the manufacturer's instructions. His-tagged BMH1 and BMH2 proteins were expressed in *Escherichia coli* DH5 $\alpha$  and purified with Ni-NTA columns (Moorhead et al., 1999). Affinity chromatography was performed with 2  $\mu$ g each of purified 14-3-3 proteins BMH1 and BMH2. After extensive washes, the bound proteins were eluted either with SDS or with 1 mM ARAAPSA peptide or 1 mM phospho- $\alpha L$  peptide (CLKPLHEKDSEpSGGGKD), and the eluates were run on SDS-PAGE and stained with Coomassie blue.

### 14-3-3 affinity chromatography

Purified BMH1 or BMH2 or BSA were coupled to Sepharose (Moorhead et al., 1999). Jurkat cells were either left untreated or stimulated with 1.5  $\mu$ M of okadaic acid, a combination of okadaic acid and the OKT3 antibody (10  $\mu$ g/ml) against the TCR, or 200 nM PDBu. The cells were lysed as described previously (Valmu et al., 1999b). The lysates were mixed with the affinity matrix for 1 h and washed extensively with 500 mM NaCl. Bound proteins were eluted with SDS and analyzed by Western blotting with the blotting  $\beta 2$  integrin antibody R2E7B.

### Coimmunoprecipitation

Human T cells were activated with OKT3 or left untreated. Cells were lysed as described previously (Valmu et al., 1999b) and lysates were pre-cleared with protein G-Sepharose. Immunoprecipitations were made with the 14-3-3 H8 antibody or control antibody (OKT3) coupled to protein G-Sepharose, and the immunoprecipitates were washed four times with lysis buffer. The bound proteins were eluted with SDS and analyzed by Western blotting with a 14-3-3 antibody (K-19) and  $\beta 2$  integrin antibody (R2E7B).

### Cell adhesion assays

Recombinant soluble human ICAM-1 or fibronectin (0.3  $\mu$ g/well) was coated on flat-bottom 96-well microtiter plates by overnight incubation at 4°C. The wells were blocked with 1% dry milk for 1.5 h at 37°C. Cells

were suspended in binding medium (DME for COS-1 and RPMI 1640 for J- $\beta$ 2.7 cells, with 40 mM Hepes, 0.1% BSA, and 1–2 mM  $\text{MgCl}_2$ ). Cells were stimulated with either 200 nM PDBu or 10  $\mu\text{g}/\text{ml}$  OKT3 or MEM-83, added to each well, and allowed to adhere for 20 (COS-1) or 30 (J- $\beta$ 2.7) min at 37°C. In inhibition experiments, cells were preincubated for 15 min with 10  $\mu\text{g}/\text{ml}$  of the blocking antibody R7E4. After incubation, unbound cells were removed by gentle washing. The binding was quantified by ELISA.

#### Immunofluorescence staining

Human T cells or J- $\beta$ 2.7 cells were seeded onto poly-lysine- or ICAM-1-coated coverslips at  $5 \times 10^5$  cells/slide in culture medium in the presence or absence of activators and incubated for 30–60 min at 37°C. For transfected COS-1 spreading assays,  $2 \times 10^5$  cells were seeded onto ICAM-1-coated coverslips and incubated for the times indicated in the figure legends. Unbound cells were gently washed away and adherent cells were fixed for 10 min with 1% formaldehyde/PBS. Cells were labeled with FITC- or TRITC-phalloidin in 0.1% saponin/1% FBS/PBS for 20–30 min, or cells were incubated with primary antibodies (mAb24, R7E4, 14-3-3 antibody K19 in PBS, or in saponin buffer) for 30 min, followed by incubation with Cy3-conjugated anti-mouse or FITC-conjugated anti-rabbit antibody. After washing with PBS, coverslips were mounted with Mowiol mounting medium and observed under a fluorescence microscope (model IX71; Olympus) and photographed with a camera (model DP70; Olympus). Images were analyzed and processed using the analysis program (Soft Imaging System GmbH) and Adobe Photoshop.

#### <sup>32</sup>P radiolabeling and cell activation, immunoprecipitation, and SDS-PAGE

<sup>32</sup>P cell labeling was done as previously described (Valmu et al., 1999b). T cells were labeled overnight and COS-1 cells were labeled for 2 h at 37°C. After labeling, the cells were activated or left untreated. The T cell activation was stopped by adding ice-cold 10 mM EDTA/PBS, and COS-1 cells were detached with 5 mM EDTA/PBS. The cells were washed and lysed as described previously (Valmu et al., 1999b). The intensity of the radiolabeled bands was quantified using the Tina 2.09c software (Raytest). The intensity of the radiolabeled bands was reported as intensity of the band minus background intensity of the lane.

Cell lysates were used for immunoprecipitation with 1–3  $\mu\text{g}$  R7E4 or IB4 for 2.5 h or overnight and coupled to protein G-Sepharose for 30 min at 4°C. The immunoprecipitates were washed extensively with decreasing detergent and salt concentrations. Bound proteins were eluted with 1% SDS, subjected to SDS-PAGE, and blotted onto polyvinylidene fluoride membranes (Millipore). Phosphopeptide mapping and manual radiosequencing of phosphopeptides have been described previously (Hilden et al., 2003).

#### Flow cytometric analysis

Cells were incubated with PBS containing 20  $\mu\text{g}/\text{ml}$  of the indicated antibodies (R7E4, MEM-83, and OKT3) on ice for 30 min. The cells were then washed with PBS and further incubated with FITC-conjugated anti-mouse IgG and subjected to flow cytometric analysis with FACScan (Becton Dickinson). For mAb24 staining, J- $\beta$ 2.7 cells or COS-cells were reacted with 10  $\mu\text{g}/\text{ml}$  mAb24 in the presence of the activating antibody 10  $\mu\text{g}/\text{ml}$  MEM-83 for 30 min at 37°C. Cells were instantly stained with FITC-conjugated anti-mouse IgG antibodies on ice for 20 min and analyzed by flow cytometry. mAb24 expression was reported as mean fluorescence intensity.

#### sICAM-1Fc binding assay

J- $\beta$ 2.7 transfectants were incubated in 25  $\mu\text{l}$  RPMI 1640, 40 mM Hepes, and 1 mM  $\text{MgCl}_2$  in the presence or absence of stimulators and 150  $\mu\text{g}/\text{ml}$  ICAM-1Fc at 37°C for 30 min. After removal of the unbound ligand by washing with PBS, the cells were incubated with FITC-conjugated anti-human IgG-Fc-specific antiserum (Jackson ImmunoResearch Laboratories) on ice for 20 min. Cells were analyzed by flow cytometry.

We thank T. Pawson for R18 constructs, C. MacKintosh for BMH1/BMH2-expressing bacteria, D. Calderwood for the talin construct, D. Cantrell for the GFP-Rap1V12 construct, and N. Hogg, V. Horejsi, and M.A. Arnaout for antibodies and cells. We would also like to thank M. Aatonen for expert technical assistance and C. Kantor for assistance with antibody production.

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